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A chronoamperometric sensor for hydrogen peroxide based on electron transfer between immobilized horseradish peroxidase on a glassy carbon electrode and a diffusing ferrocene mediator

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Abstract

A sensor has been constructed by adsorptive immobilization of horseradish peroxidase onto a glassy carbon electrode, thus permitting a sensitive chronoamperometric determination of low micromolar levels of hydrogen peroxide. The method is based on enzymatic reduction of the substrate (H_2O_2) by peroxidase and subsequent electron transfer from the glassy carbon electrode to the enzyme, via a redox mediator. To a mediator solution (0.5 mM of ferrocene monocarboxylic acid in a 0.05 M buffer solution of phosphate at pH = 7.20, 0.5 ml volume), 50 μl of the sample of H_2O_2 is injected and the current–time response is recorded with an applied potential of +50 mV. The peak height of the obtained chronoamperogram varies linearly with the final H_2O_2 concentration in the range 0.5×10^{-7}–5×10^{-6} M. The attained detection limit of the enzyme electrode is 4×10^{-8} M (1.4 ng ml^{-1}) of H_2O_2 in the final solution. The lifetime of the sensor is at least one week (about 120–160 analyses are carried out in this time). The effects of possible interferences of uric acid, ascorbic acid, haemoglobin, riboflavin, bilirubin, albumin, glutathione, D+glucose, pyruvic acid and lactate have also been studied. The use of the sensor to determine hydrogen peroxide in samples of bovine serum, heparinized blood and water is also shown.

Keywords: Chronoamperometric H_2O_2 sensor; Ferrocene mediator; Glassy carbon electrode; Horseradish peroxidase

1. Introduction

The determination of trace amounts of H_2O_2 is of practical importance in clinical, environmental and biological studies, and it is used in many other fields, such as chemical and industrial processes (e.g., wastewater treatment) [1]. Many amperometric sensors have been developed for this purpose. Widespread interest may also be due to the clinical, industrial, and hence commercial, importance of such a biosensor [2].

Analysis of biological systems, such as blood and brain fluid, is probably its main area of application, but a problem with such analyses is the presence of endogenous electroactive species, e.g., ascorbic and uric acids (AA and AU, respectively), which may oxidize directly on the electrode surface and contaminate the enzyme-mediated current [3]. Since the concentration of these interfering substances fluctuates constantly in biological tissues and fluids, it is essential to eliminate these effects.

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The biosensor systems that have received by far the greatest attention to date are enzyme electrodes [2]. For instance, horseradish peroxidase (HRP) usually catalyses the oxidation of substrates by H_2O_2 and it is a very commonly used enzyme in amperometric measurements [2,4,5].

A range of techniques exists to immobilize the enzyme at an electrode surface: e.g., covalent attachment with functional groups generated on the surface of the electrode [2], the crosslinking of the enzyme in a protein matrix using glutaraldehyde [2], trapping of the enzyme in a polymeric film support attached to solid electrodes (crosslinked redox conducting polymers can also act as effective electron-transfer mediators) [6,7], mixing with graphite in the so-called carbon-paste electrodes [8–10], adsorption [11], physical trapping of the enzyme behind a dialysis membrane [12] or even the use of tissues with a high HRP content [13].

These approaches have often been combined with the use of a macroscopic membrane in order to reduce electrode fouling and to diminish interference caused

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by electroactive species present in the sample [9,12,14].

Direct electron transfer from the electrode to the haem constituting the redox centre of HRP is mainly hindered by the insulating polypeptide network of HRP and by the spacer layer between HRP and the electrode [15]. Although some papers report there being an apparent direct electron transfer between various electrode materials and peroxidases [15,16], a mediator is usually required [5,10].

Many enzyme electrodes make use of synthetic mediators that shuttle this electron transfer and offer new advantages such as a lower necessary overpotential, thus diminishing the interferences of electroactive species usually present in physiological fluids [4,5]. The small-molecule electroactive mediators are used either free in solution [11,17] or bound to the electrode surface [5,18,19] in order to increase the rate of electron transfer between the electrode and protein.

The mediator must react rapidly with the reduced enzyme, exhibit chemically reversible behaviour, and possess a low overpotential for regeneration. Although many organometallic and organic mediators have been proposed (e.g., phthalocyanines, hexacyanoferrate(III), ruthenium oxide complexes, metalloporphyrins, quinone, tetrathiafulvalene, phenylenediamine or phenazine) [4,10,11,18], the ferrocene group has proved to be particularly important in the development of sensors involving H_2O_2 determination [5,8,9,17–19].

In this paper, a peroxidase-modified glassy carbon electrode for the chromoamperometric determination of H_2O_2 is described. The enzyme is immobilized on the carbon substrate by adsorption, and ferrocene monocarboxylic acid is used as a substrate dissolved in the sample solution. The reaction is the two-electron oxidation of peroxidase in the presence of substrate (H_2O_2), the latter being reduced to water. The ferrocene is then oxidized by the enzyme acting as an electron donor to the oxidized iron in the haem protein, the native enzyme being regenerated by electron transfer from the mediator in its reduced form.

The total charge necessary to reduce the mediator on the electrode (related to peak-height in the obtained chronoamperogram) was measured by chronoamperometry with $E_{applied} = +50$ mV versus SCE, and this change is proportional to the concentration of H_2O_2. The time for the evolution of the complete chronoamperogram is only about 20 s.

2. Experimental

2.1. Apparatus

Electrochemical experiments and chronoamperometric measurements were performed with an Inelecsa 1212 potentiostat/galvanostat interfaced with an Acer 915 computer (80286 microprocessor IBM compatible) and a Star LC-10 printer for recording the chronoamperograms. Instrument parameters of the chronoamperometric techniques, stirring and digital peak measurements were controlled by compiled BASIC programs.

All electrodes were from Methrom. A saturated calomel electrode (SCE) and Pt wire were used as reference and counter electrodes, respectively. A glassy carbon electrode (GCE, 3 ± 0.05 mm diameter) and rotating disk electrode (RDE, model 628-10, 3 ± 0.05 mm diameter) were used alternately as working electrodes. A minivoltammetric cell with a maximum working volume of 1.5 ml was used.

A digital Crison conductivimeter (model 525), with conductivity cells of $K = 1$ cm^{-1} was also used to test the ohmic resistance of solutions.

2.2. Reagents

Peroxidase (type VI-A) from horseradish was obtained from Sigma Chemical Co. Stock solutions of 1 mg ml^{-1} were prepared by dilution in a 0.1 M phosphate buffer (pH = 7.0) and stored at 4 °C in a refrigerator.

Stabilized 30% hydrogen peroxide solution was purchased from Merck. The concentration of more dilute solutions of peroxide (prepared daily) was standardized by titration with potassium permanganate.

Commercially available ferrocene monocarboxylic acid (Aldrich) was directly used. Solutions of 0.5 mM were prepared daily by dissolving in a previously deoxygenated phosphate buffer (0.05 M, pH = 7.0) after passing a nitrogen flow for 15 min.

A 5 wt.% solution of Nafion® (Aldrich, prepared from Nafion® 117 perfluorinated membrane) was diluted in a 0.9/0.1 (v/v) methanol/water mixture to obtain the coating solution containing 0.04 wt.%.

All other chemicals were of high purity (analytical-reagent grade, Merck). High-purity water was obtained from a Millipore Milli-Q water system. All sample phials were rinsed in 0.5 M nitric acid and high-purity water before use.

2.3. Preparation of the enzyme electrode

Before immobilizing the enzyme, the GCE was polished for 60 s with 0.3 μm α-Al_2O_3 (alumina) particles, washed thoroughly with water and ethanol between each polishing step, sonicated in water for 5 min, rinsed again and dried with filter paper. The peroxidase was immobilized by adsorption to cover the active carbon disk, and this was done by dipping the electrode at 4 °C for 24 h in a 10 μg ml^{-1} horseradish peroxidase (type VI) solution in phosphate buffer. The electrode was left at room temperature and conditioned with five measurements before use. It was stored at 4 °C in a

0.1 M phosphate buffer (pH=7.02) when not in use. The enzymatic electrode was useful for at least one week.

3. Results and discussion

3.1. Analytical procedure

A solution of mediator (ferrocene monocarboxylic acid, 0.5 mM) and buffer (phosphate 0.05 M, pH=7.02) was deoxygenated with N_2 for 5 min in a minivoltammetric cell. The total volume of the former solution (mediator in phosphate buffer) is 0.5 ml. After maintaining the rest potential (E_{rest}) of +25 mV for 10 s, a potential $E_{app}=+50$ mV was applied (step height +25 mV) and then 50 μl of the sample solution with H_2O_2 (containing from 0.5×10^{-6}–5×10^{-5} M of H_2O_2) was injected with an Eppendorf micropipette within an interval of 10 s (the dilution factor is 1/11). While maintaining $E_{app}=+50$ mV for 50 s, the current–time response (chronoamperogram) was recorded.

Typical chronoamperometric (current–time) response curves on addition of sample containing H_2O_2 are shown in Fig. 1. The fundamental reaction is the enzymatic reduction of H_2O_2 to water by horseradish peroxidase with the ferrocene acting as an electron donor; the ferricinium ion is reduced at the electrode, then the native enzyme is regenerated by subsequent electron transfer from an electrode to the peroxidase via the ferrocene mediator.

3.2. Preliminary assays

A number of ferrocene derivatives with a range of redox potentials (usually between 100 and 450 mV versus SCE) have been evaluated in the literature as oxidants, mainly for HRP and glucose oxidase [4,5,19]. They usually have a well-behaved redox couple, exhibiting rapid electron-transfer reactions and properties (such as solubility and redox halfwave) that are easily modified by substitution [19].

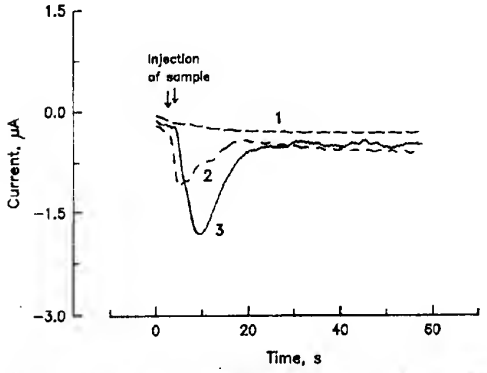

Fig. 1. Typical chronoamperometric responses to additions of hydrogen peroxide solutions: 1, blank solution (---); 2, after injection of 50 μl of a 5.4 μM H_2O_2 solution (----), the concentration of final solution being 0.5 μM H_2O_2; 3, after injection of 50 μl of a 9.8 μM H_2O_2 solution (——), the concentration of final solution being 0.9 μM H_2O_2.

Ferrocenemonocarboxylic acid (FC) was selected for this work because of its good ability to mediate electron transfer between a number of flavoprotein oxido-reductases, particularly HRP and H_2O_2 [5], and its poor solubility in water (a good characteristic for its immobilization). HRP reduces H_2O_2 to water and ferrocene can then act as an electron donor to the oxidized iron in the haem protein. The ferricinium ion can be reduced at the electrode, the charge involved being proportional to the concentration of hydrogen peroxide.

Cyclic voltammetry was used first of all in order to assess the ability of the ferrocene to act as a mediator in a variety of electrode substrates. Initial experiments in a batch system containing a dissolved mediator, H_2O_2 and HRP were carried out by recording a series of cyclic voltammograms between 0 and +500 mV at scan rates of 10–200 mV s^{-1}.

FC gave consistent voltammograms, controlled solely by diffusion, with reversible one-electron electrochemical behaviour ($\Delta E_p\approx60$ mV, $i_p/(v)^{1/2}\approx$ constant) with all the following Metrohm disk electrodes (3 ± 0.5 mm diameter): GC, Au, Pt and Ag. Introduction of peroxidase had no discernible effect upon their electrochemistry.

However, on addition of H_2O_2 to a solution of FC and HRP, steady-state voltammograms of the EC (chemical following electrochemical electrode process) mechanism [19] were observed at a scan rate of 10 mV s^{-1}: no peaks were observed and a catalytic current flowed at reducing potentials, which implies a catalytic regeneration of the oxidized mediator by peroxidase in the presence of hydrogen peroxide. This effect is observed in Fig. 2. Steady-state behaviour provides preliminary evidence that the kinetics of the HRP-catalysed oxidation of the mediator are rapid.

Such electrochemical behaviour was found with both carbon and metallic electrode substrates, and so a GC substrate was selected as the most effective for the subsequent enzyme immobilization. Also the carbon electrode gave the greater constants for $i_p/(v)^{1/2}\approx$ constant, which implies that it gave the best sensitivity (charge-transfer) for the same concentration of FC compared with the others.

The performance of some substituted ferrocene mediator couples (including FC) has already been evaluated by several authors using cyclic voltammetry and diverse enzyme substrates [4,5,19]. Similar behaviour is reported, and good charge-transfer rate constants were calculated in all cases.

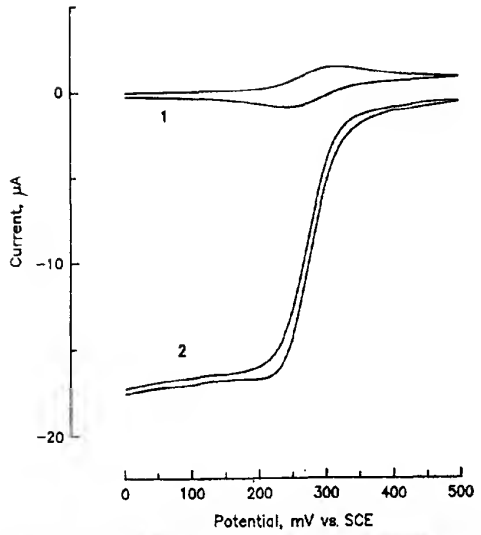

Fig. 2. Cyclic voltammograms of: 1, the ferrocene mediator (0.25 mM) solution with HRP (5 $\mu g\ ml^{-1}$); 2, with addition of hydrogen peroxide (1 mM). Potential scan rate 5 $mV\ s^{-1}$.

A complete electrochemical study of this system (H_2O_2, FC and HRP) was performed by cyclic voltammetry with a GCE substrate and by the use of Nafion as a polymer network to exclude interferences. Three initial attempts at determining hydrogen peroxide were assayed:

(1) immobilization of peroxidase and ferrocene monocarboxylic acid on the GCE and covering it with an excluding polymer (Nafion);

(2) immobilization of the mediator with and without Nafion and solubilized peroxidase;

(3) immobilization of the peroxidase on the electrode and adding the mediator to sample solution.

Coating of working electrodes with polymer permselective membranes is a common procedure introduced to circumvent the adsorption of surface-active compounds onto a planar GC surface [20,21]. The attractive features of Nafion (chemical inertness, non-electroactivity, hydrophilic and insoluble in water) have been particularly useful in voltammetry [20,21].

The first attempt to cover the enzyme layer with an anionic exclusion polymer such as Nafion was carried out. The very thin films of the Nafion coat allowed rapid electrode response to the mass transport of the analyte, there being a compromise between the unhindered transport of the H_2O_2 and the exclusion of organic matter or interfering substances present in the sample solution. The aim was to exclude interfering readily oxidizable species such as ascorbic and uric acids (usually present in biological fluids at concentrations of 5–20 mg/100 ml), which cannot diffuse through the polymer. The effectiveness of the Nafion in excluding large molecules can be observed in the cyclic voltammograms of Fig. 3.

The electrode was covered quickly and simply as described previously [20,21] by applying 7 μl of a 0.04% (m/v) Nafion solution to the GC surface with a microlitre pipette. The thickness can be controlled by varying the concentration of the Nafion solution and/or the volume of this solution. Previously the FC and HRP were co-immobilized (in the order cited) on the GC substrate as described.

Although the modified electrode with Nafion shows good selectivity to AU and AA in the determination of H_2O_2 (see voltammograms of Fig. 3), nevertheless, desorption of the enzyme and FC was observed, together with losses (solubilization) from the electrode after several measurements. This was observed when attempts to immobilize the enzyme and/or FC and also covering with Nafion were carried out. The reason for this is that Nafion solubilizes the adsorbed peroxidase and ferrocene monocarboxylic acid on the electrode, and it also denaturizes the enzyme because it is dissolved with methanol.

Although the Nafion effectively excluded interfering molecules such as AA and AU, it was not subsequently used. Unfortunately a modified electrode prepared by adsorption of FC only (second attempt) was also not successful due to its quick solubilization after 10–15 measurements and the expense because of the enzyme loss after the H_2O_2 determination.

In both cases the mediator (ferrocene) was adsorbed onto the graphite substrate by depositing 10 μl of 0.05 mM FC solution in toluene on the electrode tip, and

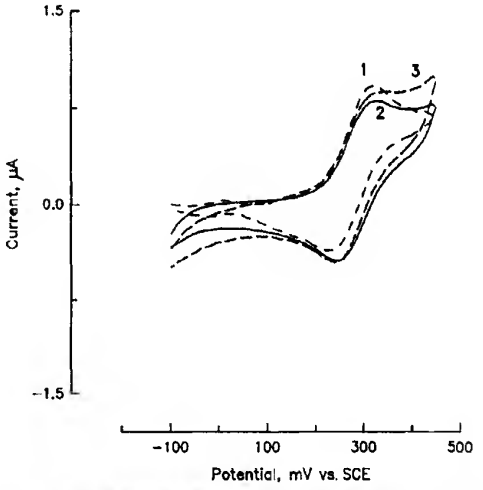

Fig. 3. Cyclic voltammograms of the ferrocene mediator ([FC] = 0.20 mM in all cases) with: 1, bare GCE (----); 2, GCE modified by covering with 7 μl of 0.04% (m/v) of Nafion (——); 3, same as before but with addition of uric acid (5×10^{-3} M) and ascorbic acid (5×10^{-3} M) solutions (– – –).

leaving it to evaporate to air. Besides, it was electrochemically observed that FC is best solubilized in a deoxygenated phosphate buffer rather than in water or methanol.

The third attempt was the best, with mediator added to the sample solution. The procedure for the immobilization of the enzyme is described in Section 2. The lifetime of the enzyme electrode was at least one week (approximately 150–200 measurements can be made in that time) if correctly stored at 4 °C in a refrigerator in a 0.1 M, pH=7 phosphate buffer.

Therefore, our method offers the following advantages: immobilization of HRP to prevent the loss of this expensive reagent; good electron transfer between the immobilized HRP and the soluble mediator; a low redox potential (below +100 mV) to reduce the ferricinium; and reduced interference by electroactive species such as AA or AU.

3.3. Chronoamperometric determination of hydrogen peroxide

The electrochemical determination of H_2O_2 is based on the following enzymatic reaction:

$$H_2O_2 + 2FC + 2H^+ \xrightarrow{\text{Peroxidase}} 2H_2O + 2FC^+$$

where HRP catalyses the reduction of substrate (hydrogen peroxide) mediated with the electron donor FC. The reduction of the oxidized ferricinium thus constitutes an indirect means of determining the concentration of H_2O_2 involved in the reaction.

Samples were analysed as described previously (refer to Section 3.1 for details). Samples with different concentrations of H_2O_2 were injected using a micropipette and the chronoamperometric response arising from the reduction of ferrocene (current–time) was recorded. Fig. 1 shows typical chronoamperograms following this procedure, the peaks obtained being clearly dependent upon the concentration of hydrogen peroxide introduced into the system. In these chronoamperograms, the maximum catalytic current (peak-height) was plotted against the H_2O_2 concentration.

The applied potential (+50 mV) was just below 225 mV from the potential of the ferrocene redox couple, thus avoiding high overpotential and background current. A second step of ΔE_p (pulse potential) = −50 mV (potential after pulse = 0 mV) with t_p (pulse time) = 30 s was finally applied in order to condition the electrode for the next measurement. This obviously recorded an inverse chronoamperogram, which is not important for analytical purposes.

Chronoamperometry as a pulse-mode amperometry was used, in contrast with the normal d.c. amperometry mode where the potential is continuously kept at the same reducing potential. This implies some advantages, like reduced electrode fouling or passivation [18]. Pulse potential and the measurement of current with time (a non-stationary method) is also advisable when the charge transfer in the electrode is coupled with chemical reactions or adsorption processes.

Stirring of the sample solution was not necessary because of the small volume (0.5 ml) and the rapid homogenization when injecting the solution with H_2O_2. Convection during injection was avoided as much as possible by depositing the solution uniformly with care, in order to improve the precision and shape of the chronoamperogram. In consequence the obtained signals are controlled solely by diffusion.

3.4. Analytical performance

A series of standard solutions was injected in triplicate to test the linearity of the calibration graph. The current response was linear with the concentration of H_2O_2 in the final solution ranging from 0.5×10^{-7} to 5×10^{-6} M (slope 1.88 $\mu A\ \mu M^{-1}$, correlation coefficient $r=0.9990$, $n=10$).

The reproducibility of the electrode calculated from a solution of 7×10^{-7} M H_2O_2 with the described experimental procedure, expressed as a relative standard deviation, was 5.92% ($n=10$ repeated measurements).

The slope and linearity of the calibration graph remained near reproducible a week after the electrode fabrication, 120–140 measurements being taken in this time, and hence the electrode can be used for at least one week after HRP immobilization. Nevertheless, because slight variations in slope could occur with time, it is advisable to use the standard additions method for application of the enzyme electrode. Care should also be taken to avoid enzyme saturation with high concentrations of substrate (H_2O_2), which obviously produces anomalous results. The maximum advisable concentration of H_2O_2 was about 8×10^{-6} M. The attained detection limit of the enzyme electrode was 4×10^{-8} M (1.4 ng ml^{-1}) of H_2O_2 in the final solution.

3.5. Interferences

The effect of substances that might interfere with the response of the enzyme electrode was examined. Table 1 summarizes the interference effect among various interfering species commonly present in both the biological and the water samples used.

For the study of interference the standard additions methods was used: 50 μl of two sample solutions with H_2O_2 alone and H_2O_2 + interferent were successively injected in duplicate. In all cases the H_2O_2 concentration was 7×10^{-7} M.

The interference mechanism could be the direct oxidation/reduction of species on the electrode, inhibition of the enzyme or chemical reaction with ferrocene and/or hydrogen peroxide.

Table 1
Effect of potentially interfering substances on the chronoamperograms for the determination of H_2O_2: ratio to original signal with respect to 7×10^{-7} M of H_2O_2 that does not interfere

Interferent	Ratio of concentrations
Albumin	5000/1
Haemoglobin	1000/1
Glutathione	1000/1
Bilirubin	1000/1
D+glucose	1000/1
Riboflavin	1000/1
Lactic acid	500/1
Ascorbic acid	100/1
Uric acid	1000/1
Pyruvic acid	500/1

Table 2
Results on the determination of H_2O_2 in blood and water samples. Concentrations are expressed as mean ± s.d. ($n=4$)

Sample	Found concentrations ($\mu g\ ml^{-1}$)
Bovine whole blood (heparinized)	1.98 ± 0.06
Bovine serum	1.00 ± 0.08
Bovine plasma	1.15 ± 0.07
Drinking water	0.87 ± 0.05

A common interferent is ascorbate, which is readily oxidizable and present at high concentrations in blood and other biological fluids. However, an important feature of mediated electrodes is that they can be applied where interference at positive potentials may pose a problem. This sensor is operated at an overpotential that is too low to cause direct oxidation of ascorbate, which greatly reduces the capacity of ascorbate and other substances to interfere with it. As can be seen in Table 1, for instance, H_2O_2 can be detected even in the presence of large concentrations of other naturally reducing substances such as ascorbic, lactic and uric acids.

3.6. Applications

The sensor was applied to determine hydrogen peroxide in drinking and natural (river) water and bovine blood samples. No pretreatment of the samples was required due to the sensitivity and selectivity of the method. The standard additions method was used: 50 μl samples (diluted with water to about one tenth or with added H_2O_2 solution) were successively injected. All measurements were carried out in duplicate. Results are summarized in Table 2.

4. Conclusions

The construction of an enzymatic sensor by adsorptive immobilization of peroxidase onto a glassy carbon electrode, based on diffusing ferrocene mediator, provides a modified electrode with excellent analytical performance to determine hydrogen peroxide. It is easy to prepare and gives good long-term stability.

The immobilization of the enzyme enables this expensive reagent to be reutilized economically, the lifetime of the electrode being about one week (stable signals for at least 150–180 measurements).

Problems with convection (diffusion) on injecting the sample solution are minimized because of the small volume of the ferrocene solution.

The analytical utility of the sensor in the detection of hydrogen peroxide has been demonstrated in water and blood samples with no necessary pretreatment. Ascorbic and uric acids did not interfere up to a ratio of 100/1 with respect to the hydrogen peroxide concentration.

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References

[1] D. Price, P.J. Worsfold and R.F.C. Mantoura, Hydrogen-peroxide in the marine-environment – cycling and methods of analysis, *Trends Anal. Chem., 11* (1992) 379–384.
[2] P. Vadgama and P.W. Crunp, Biosensors: recent trends. A review, *Analyst, 117* (1992) 1657–1670.
[3] J.P. Lowry and R.D. O'Neill, Homogeneous mechanism of ascorbic acid interference in hydrogen peroxide detection at enzyme-modified electrodes, *Anal. Chem., 64* (1992) 453–456.
[4] J.E. Frew, M.A. Harmer, H.A. Hill and S.I. Libor, A method for estimation of hydrogen peroxide based on mediated electron transfer reactions of peroxidases at electrodes, *J. Electroanal. Chem., 201* (1986) 1–10.
[5] M.J. Green and H.A.O. Hill, Amperometric enzyme electrodes, *J. Chem. Soc., Faraday Trans. 1, 82* (1986) 1237–1243.
[6] J. Wang, Y. Lin and L. Chen, Organic phase biosensors for monitoring phenol and hydrogen peroxide in pharmaceutical antibacterial products, *Analyst, 118* (1993) 277–280.
[7] P.N. Barlett, P. Tebbutt and H. Tyrrell, Electrochemical immobilization of enzymes. 3. Immobilization of glucose oxidase in thin films of electrochemically polymerized phenols, *Anal. Chem., 64* (1992) 138–142.
[8] P.D. Sanchez, A.J.M. Ordieres, A.C. Garcia and P.T. Blanco, Peroxidase ferrocene modified carbon paste electrode as an amperometric sensor for the hydrogen peroxide assay, *Electroanal., 3* (1991) 281–285.
[9] J. Wang, L. Angnes and C. Liang, Electrocatalysis and amperometric detection of organic peroxides at modified carbon-paste electrodes, *Talanta, 38* (1991) 1077–1081.

[10] S.A. Wring and J.P. Hart, Chemically modified, carbon-based electrodes and their application as electrochemical sensors for the analysis of biologically important compounds, *Analyst, 117* (1992) 1215–1229.
[11] P. Dominguez, P. Tuñon, J.M. Fernandez, M.R. Smyth and R. O'Kennedy, Flow-injection analysis of hydrogen peroxide using a horseradish peroxidase-modified electrode detection system, *Electroanal., 2* (1990) 303–308.
[12] J.L. Marty, K. Sode and I. Karube, Biosensor for detection of organophosphate and carbamate insecticides, *Electroanal., 4* (1992) 249–252.
[13] J. Wang and N. Naser, Horseradish-root tissue tubular reactor for flow injection detection of hydrogen peroxide, *Anal. Lett., 25* (1992) 999–1009.
[14] F. Cheillan, A. Albano and M. Mascini, Amperometric biosensors for measurement of cholesterol and application to clinical samples, *Annal. Chim., 81* (1991) 673–691.
[15] T. Tatsuma and T. Watanabe, Peroxidase model electrodes – heme peptide modified electrodes as reagentless sensors for hydrogen peroxide, *Anal. Chem., 63* (1991) 1580–1585.
[16] L. Gorton, G. Jonsson, E. Csoregi, K. Johansson, E. Dominguez and G. Marko-Varga, Amperometric biosensors based on an apparent direct electron-transfer between electrodes and immobilized peroxidase – plenary lecture, *Analyst, 117* (1992) 1235–1241.
[17] Y. Zhang and G.S. Wilson, Electrochemical oxidation of H_2O_2 on Pt and Pt–Ir electrodes in physiological buffer and its applicability to H_2O_2-based biosensors, *J. Electroanal. Chem., 345* (1993) 253–271.
[18] H. Gunasingham, T. Chin-Huat and H.M.J. Ng, Pulsed amperometric detection of glucose using a mediated enzyme electrode, *J. Electroanal. Chem., 287* (1990) 349–362.
[19] A.E.G. Cass, G. Davis, G.D. Francis, A.O. Hill, W.J. Aston, I.J. Higgins, E.V. Plotkin, L.D.L. Scott and A.P.F. Turner, Ferrocene-mediated enzyme electrode for amperometric determination of glucose, *Anal. Chem., 56* (1984) 667–671.
[20] J.C. Vidal, G. Cepria and J.R. Castillo, Models for studying the binding capacity of albumin to zinc by stripping voltammetry, *Anal. Chim. Acta, 259* (1992) 129–138.
[21] J.C. Vidal, R.B. Viñao and J.R. Castillo, Binding capacity of casein to lead and voltammetric speciation of lead in milk with a nafion coated electrode, *Electroanal., 4* (1992) 653–659.

Biographies

Juan C. Vidal received his Ph.D. degree in analytical chemistry in 1984 from the University of Zaragoza (Zaragoza, Spain). His Ph.D. thesis focused on the extraction into non-aqueous solvents of complexed metals and the determination of these metals directly in organic solvents by molecular absorption spectrophotometry and anodic stripping voltammetry. For the past five years his research has dealt with the application of analytical stripping voltammetry for speciation of metals in milk or blood samples, electroanalytical characterization of superconductor materials or electrolytic preconcentration on L'vov platforms before atomization. His current research work focuses on the development of enzymic biosensors and electrochemical detection by chemically modified electrodes. He has been assistant professor of analytical chemistry at Zaragoza University since 1982, and titular professor at this University since 1986. He is the author of about 40 publications in international journals, and has collaborated in about six research projects of the DGICYT (Spanish Research Department).

M.A. Yague received her B.S. degree in analytical chemistry from the University of Zaragoza (Zaragoza, Spain) in 1990. She collaborated in the Analytical Chemistry Department for two and a half years (from 1990 to mid 1993), working with analytical voltammetry and enzymic electrodes for amperometric measurements. Her current work is carried out in the industrial field and deals with the analytical determination of metals.

J.R. Castillo received a Ph.D. in analytical chemistry from the University of Zaragoza (1974). Since 1982 he has been an analytical full professor in the Department of Analytical Chemistry, Faculty of Sciences. His main research interest is related to analytical atomic and molecular spectroscopy and methods to obtain analytical spectroscopic measurements from gas and solid samples. Recently, a new target has been the research work on sensors, mainly biosensors based on enzymic reactions, electrochemical processes and the surface plasmon resonance effect. He is the author of more than 110 papers in international journals and books, and conference papers at international congresses.